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(54) Title: RAPID INDUCIBLE BETA-LACTAMASE SCREEN TEST

## (57) Abstract

In an assay for determination of beta-lactam antibiotic resistance in a target bacterial strain, the strain is grown in the presence of both a beta-lactamase inducing antibiotic and a beta-lactam indicator antibiotic which kills or inhibits the growth of bacteria unable to hydrolyze beta-lactam antibiotics. Growth, indicative of drug resistance in the target strain, is monitored by detecting a fluorophor released by the enzymatic cleavage of a metabolizable fluorogenic compound.

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## RAPID INDUCIBLE BETA-LACTAMASE SCREEN TEST

## BACKGROUND OF THE INVENTION

Antimicrobial susceptibility tests are among the most important tests performed in the clinical microbiology laboratory. Administration of antibiotics to patients whose infections are caused by bacteria resistant to those antibiotics seriously compromises and delays effective therapy. Resistance to antibiotics is mediated by several mechanisms. In the case of antibiotics containing a beta-lactam ring structure, such as the penicillins, cephalosporins, carbapenems, cephemycins, and monobactams, resistance is conferred by beta-lactamases, which hydrolyze the beta-lactam ring, thereby inactivating the antibiotic.

The genes encoding the beta-lactamases may be constitutively expressed by plasmids carried by the bacterial host, or may reside in the bacterial chromosome. Class I beta-lactamases are of the latter type and are induced following exposure to a beta-lactam antibiotic. These enzymes are produced in finite but very low amounts in the uninduced state. Class I beta-lactamases include the cephalosporinases, which inactivate all beta-lactam antibiotics including penicillins, cephalosporins, cephemycins, and monobactams. Virtually all strains of Citrobacter freundii, Enterobacter spp., Morganella morganii, Proteus vulgaris, Providencia spp., Pseudomonas aeruginosa, and Serratia spp. contain class 1 beta-lactamases.

It is known that class I beta-lactamases are induced to varying degrees by different beta-lactam antibiotics, as summarized by Sanders, et al., Eur. J. Clin. Microbiol., 6: 435 (1987). This means that

some antibiotics such as cefoxitin and imipenem are excellent inducers, resulting in production of high levels of beta-lactamases. Others, however, such as ticarcillin, ceftazidime, mezlocillin, piperacillin, 5 carbenicillin and ceftriaxone are not particularly good inducers of enzyme. Since the level of enzyme is too low to quantitate in the repressed (uninduced) state, assays in the prior art have adopted a format in which beta-lactamase is first induced with an 10 inducing antibiotic, and then the organism is challenged with the indicator antibiotic.

One such assay has been described by Sanders, et al., *Antimicrobial Agents and Chemotherapy*, 15:792 (1979) for an agar dilution technique. In this assay 15 a Petrie dish is overlayed with agar containing the pregrown target organism. A first disk impregnated with the inducing antibiotic is placed on the agar surface. A second disk impregnated with the indicator antibiotic is placed about 10 millimeters 20 from the first disk. The Petrie dish is incubated for some 18-24 hours.

As the bacterial lawn grows up, the antibiotics diffuse from the disks into the surrounding agar. If 25 the organism is susceptible to the indicator antibiotic, a completely round clear zone of inhibition is observed. If the organism is induced, the zone of inhibition is flattened on the edge between the two antibiotic disks. The explanation 30 for the result is that there is a diffusion race in which the embedded incipient colonies of the target organism closer to the inducing antibiotic become resistant through induction of the protective beta-lactamase before the indicator antibiotic has time to

reach them. Figure 1 of the Example hereinbelow illustrates this test.

5 Sanders, et al., *J. Infect. Dis.*, 154: 792 (1986) described an alternate method for detection of beta-lactam resistant organisms, in which the target strain is first grown up in the presence of an inducing antibiotic. The cells are collected, lysed, and beta-lactamase activity is measured in the supernatant.

10 Mett, et al., *Eur. J. Clin. Microbiol.*, 7: 669 (1988) described a relatively rapid test in which bacteria carrying a gene for inducible beta-lactamase expression are inoculated into microtiter plates for determination of the minimum inhibitory concentration. After 4 hours of incubation, a chromogenic beta-lactamase substrate is added and changes in color are monitored over the next three hours of incubation. A similar approach was reported by Jarlov, et al., *Acta Pathol. Microbiol. Immunol. Scand.*, 94: 415 (1986).

15 20 25 There have been several studies in which combinations of inducing and noninducing beta-lactam antibiotics have been utilized. See, for example, Pederson, et al., *J. Antimicrob. Chemother.*, 19: 101 (1987). The object of making these combinations is to assess a synergistic potential of the combinations against particularly intractable bacterial strains.

30 In general, the current methods of determining beta-lactam susceptibility and resistance, namely, agar dilution, broth microdilution, Kirby-Bauer, all fail to detect drug resistance in certain gram-negative bacteria, as discussed by Jorgensen, *Chemioterapia*, 4: 7 (1985). The known methods all require a pregrowth step in which the strain is grown

in broth or on a plate under conditions in which the organism is exposed only to the inducing antibiotic. This step is followed by a challenge in the presence of the an indicator antibiotic or direct assay of enzymatic activity. These approaches require pure culture inoculation and growth, and involve up to 24 hours incubation.

#### SUMMARY OF THE INVENTION

In the assays of beta-lactam antibiotic resistance in the prior art, a great deal of time is lost because a growth step is required prior to assay which takes several hours. The assay itself may then require some further several hours of incubation. Even these tests will fail to detect a bonafide resistant organism, because of the very low numbers of a mutant resistant strain which may be present. Accordingly, it an object of the present invention to provide a rapid and highly reliable assay of beta-lactam antibiotic resistance.

It is a further object to provide a simple assay which requires no handling steps involving direct enzyme assay requiring separate addition of reagents. It is a still further object of the invention to provide an assay which can utilize existing instrumentation to read and interpret results in an automated format.

In the present assay for rapid determination of beta-lactam antibiotic resistance, the target strain in pure culture is inoculated into growth media containing both a beta-lactamase inducing antibiotic and a beta-lactam noninducing indicator antibiotic, which would kill or inhibit it in the uninduced state. A metabolizable fluorogenic compound is added either in the media prior to inoculation, or after a

period of growth. A resistant organism or an inoculum containing a resistant organism will grow in the media, whereas a susceptible organism will not grow.

5 Metabolism of an essential nutrient-containing fluorogenic compound releases the fluorophor which is detected by conventional fluorimetry. A susceptible organism, in which the protective beta-lactamase is not induced, will not grow, and increase in 10 fluorescence of the sample will be noted.

10 The present invention also provides a kit comprising one or a plurality of incubation vessels containing a dried, reconstitutable first inducing beta-lactam antibiotic, a second dried 15 reconstitutable indicator noninducing beta-lactam antibiotic, and a metabolizable fluorogenic compound.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

20 In the present assay, a bacterial strain of unknown susceptibility to beta-lactam antibiotics is assayed for drug resistance. Since the chromosomally encoded beta-lactamase genes exhibit induced expression, challenge of the organism with the antibiotic directly may yield false susceptibility results. Also, the patient sample may contain a 25 number of different organisms, and within even an individual isolate, there may be small numbers of mutant organisms, either constitutively positive or negative for the enzyme, or which are mutant for the antibiotic binding site.

30 The sample is inoculated into growth media containing both an inducing and an indicator noninducing beta-lactam antibiotic. Applicants consider adding growth media already inoculated with a sample to the inducing and indicator antibiotics

either dried or resuspended in a compatible buffer to be the equivalent of inoculating growth media containing the two antibiotics, so long as both drugs are present simultaneously during incubation.

5           Inoculations should result in a final concentration of organisms of from about  $1 \times 10^5$  CFU/ml to  $1 \times 10^6$  CFU/ml. The media may be any defined media capable of supporting the growth of the species of organism so inoculated. For most gram-negative isolates, Mueller-Hinton broth is preferred.

10           The inducing antibiotic is selected from the group comprising the beta-lactam antibiotics which have been shown empirically to cause induction of the beta-lactamase gene with concomitant high level expression of the enzyme.

15           The scientific basis for the observation that some of the beta-lactam antibiotics are better inducers of beta-lactamase is not presently known. One possible mechanism is a greater affinity of a particular drug for the repressor protein, or a binding interaction which enhances the conformational effect on the repressor which controls expression of the operon. There does not, however, appear to be a correlation between the inducer function and the minimum inhibitory concentration (MIC).

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25           Inducer antibiotics include cefoxitin, imipenem, sulbactam and clavulanic acid; cefoxitin is preferred. The concentration of inducer may be varied; 0.1-20 mcg/ml is the general range of concentration with 1.0 to 10 mcg/ml being preferred.

30           Indicator antibiotics include ticarcillin, ceftazidime, mezlocillin, piperacillin, carbenicillin, and ceftriaxone; ticarcillin and ceftazidime are preferred. The concentration of

indicator may also be varied; 0.1 - 30 mcg/ml is the general range of concentration with 1.0 to 15.0 mcg/ml being preferred.

5 Other combinations of antibiotics having inducer and indicator properties may be selected from the groups of beta-lactam antibiotics set forth in Table 1 hereof. The exact concentrations for the respective antibiotics must be determined empirically, but the essential element of the present method is that the 10 inducer and indicator drugs be present simultaneously during the incubation step. In general, the assay should be conducted at or near the minimum inhibitory concentration of the indicator drug, which is determined empirically by running a drug dilution 15 series in the presence of the indicator antibiotic.

It may also be found desirable to combine two or more inducer and indicator antibiotics in the media to be inoculated. Such combinations are considered to be the equivalents of pairwise combinations. The 20 essential element is that both the inducer and indicator functionalities be present simultaneously during incubation.

It is preferred that a metabolizable fluorogenic compound be incorporated into the growth media into 25 which the patient sample is inoculated. Known fluorogenic compounds include alanine-7-amido-4-methyl-coumarin and 4-methylumbelliflferone compounds. Alanine-7-amido-4-methyl-coumarin is preferred because most gram-negative organisms are capable of 30 secreting an enzyme capable of hydrolyzing the alanine as a source of the metabolite. Cleavage of the alanine results in release of the fluorophor, which can be detected by conventional fluorimetry. In this assay format a two-fold increase in

5                   fluorescence compared to controls is deemed a positive test. Incorporating the fluorogenic compound during incubation is desirable since the cumulative effect of the release of the fluorophor gives an earlier positive determination. Bacterial 10               growth can be measured in other ways, such as by nephelometry, spectroscopy, pH change, or by determination of metabolic by-products. By these parameters, a two-fold increase of signal over background is deemed a positive test.

10               The present invention also contemplates a kit containing reagents ready for use. The kit comprises one or a plurality of incubation vessels containing dried reconstitutable antibiotics comprising at least 15               one inducing beta-lactam antibiotic, at least one beta-lactam indicator noninducing beta-lactam antibiotic, and a metabolizable fluorogenic compound. In preparing the incubation vessels it was found that air drying, e.g. passing air over the vessel compartments is preferable to lyophilizing because the antibiotics are more readily reconstituted with a 20               buffer or growth media after drying. Air drying is also less expensive than freeze drying.

25               Any instrument capable of measuring fluorescence may be utilized in practicing the present assay. Preferably an instrument is used which can monitor fluorescence during the course of the incubation. A system having a heated chamber maintained at physiological temperature, which can simultaneously 30               monitor fluorescence of at least 96 samples contained in a microtiter plate is especially desirable. One such system is the Baxter autoSCAN®-W/A instrument.

Other advantages of the present assay will be apparent from the Example which follows:

## EXAMPLE

A total of 459 gram-negative organisms were tested on the rapid  $\beta$ -lactamase screen panel. Of these, 299 organisms belong to species known to contain class 1  $\beta$ -lactamases (Group 1, Table 1); the remaining 160 isolates represent species whose  $\beta$ -lactamase genes are not known to be inducible (Group 2, Table 1).

Table 2 gives examples of mutant and wild-type strains. Results for Ent. cloacae and Ps. aeruginosa with ceftazidime (Caz) and ceftazidime plus cefoxitin (Caz w/Cfx) are shown. Results for Cit. freundii and Ent. cloacae with ticarcillin (Ti) and with ticarcillin plus cefoxitin (Ti w/Cfx) are also given. The results for a mutant (mut) strain, Ent. cloacae (#498), are given where the percent fluorescence in the control wells were already high; therefore, induction was undetectable. Typical positive induction results are given for a wild-type (w.t.) strain, Ent. cloacae #20-8, where the percent fluorescence in Caz w/Cfx was 47% versus 5% in Caz alone. Even though cefoxitin was added to all concentrations of ceftazidime and ticarcillin, induction was frequently observed only in the wells containing the lowest concentrations of the antibiotics. Similar inductions can be observed in all examples of Table 2.

Summaries of induction of  $\beta$ -lactamase, expressed as a constriction of the zone of inhibition to mezlocillin, as tested by the K-B method, or increased resistance to ticarcillin, ceftazidime or to both antibiotics (rapid  $\beta$ -lactamase screen) are given in Table 3 for organisms of groups 1 and 2. The overnight K-B (Kirby-Bauer disk approximation

test) method detected  $\beta$ -lactamase induction in 109/299 isolates (36%). The rapid method of the present invention detected induction of  $\beta$ -lactamase in 68/299 isolates (23%). Induction of resistance to both ticarcillin and ceftazidime, or to either was observed with Cit. freundii, Ent. cloacae and Ent. aerogenes. Resistance to ceftazidime only was observed with Ps. aeruginosa isolates. The rapid method could detect induction in these four species as successfully as the overnight method. The rapid method could not detect induction in Prt. vulgaris, Ser. marcescens and Aer. hydrophila. As would be expected, both methods did not detect induction in isolates of group 2 taxa.

The disk constriction method has been designed to help predict which organisms may develop in vitro resistance to  $\beta$ -lactam antibiotics, thereby to improve the choice of drug for therapy. However, expression of the genes both in vitro or in vivo is not completely understood. Although it is assumed that resistance to all  $\beta$ -lactam antibiotics is induced, the in vitro expression of such resistance to the various antibiotics is not identical. Both choice of inducer and choice of test-antibiotic may affect the results. Using the reference method, cefoxitin, clavulanic acid and imipenem were shown to be good inducers. In this method, organisms are preincubated with inducer, followed by centrifugation, lysis of the bacterial cells and detection of  $\beta$ -lactamase induction by very sensitive detection of enzymatic activity towards cephalothin.

Using the K-B method cefoxitin has been shown to be a good inducer and many of the  $\beta$ -lactam antibiotics have been shown to be suitable indicators

for detection of induction. The present study shows that cefoxitin is a good inducer for ca. 50% of the strains of Ent. cloacae and Ps. aeruginosa, induction being detected by both conventional and rapid methods. Induction was detected in Xan. maltophilia by the rapid method only. Induction was detected in Aer. hydrophila, Ser. marcescens and members of the tribe Proteae by the disk method only. These isolates were susceptible to cefoxitin and the concentration of the antibiotics used in the rapid  $\beta$ -lactamase screen test did not allow growth in sufficient quantity to be detected by the rapid method.

The rapid method was comparable to the disk approximation test in the detection of induction in Cit. freundii, Ent. aerogenes, Ent. cloacae, and Ps. aeruginosa, as well as showing no induction of any isolates of taxa in Group 2. This rapid method was better than the K-B disk approximation method for detection of induction in Xan. maltophilia. The isolates in which induction could not be detected could be divided into two categories. Those which were already resistant to the indicator antibiotics and further induction was undetectable, and those in which, even though susceptible to the indicator, induction could not be demonstrated.

The described method is the first successful rapid detection of inducible class 1  $\beta$ -lactamases. The rate of success for the majority of taxa tested is comparable to that of the overnight method.

Table 1The  $\beta$ -Lactam Family of Antibiotics<sup>a</sup>

	<u>Penicillins</u>	<u>Cephalosporins</u>	<u>Cephamycins</u>
	Benzylpenicillin	Cephalothin	Cefoxitin
5	Methicillin	Cefamandole	Cefotetan
	Ampicillin	Cefuroxime	Cefmetazole
	Carbenicillin	Cefotaxime	
	Mezlocillin	Ceftriaxone	
	Piperacillin	Ceftazidime	
10	<u>Carbapenems</u>	<u>Monobactams</u>	
	Imipenem	Aztreonam	

<sup>a</sup>Limited examples in each group are given

Table 2

Group 1 Class 1 $\beta$ -lactamase Producers		Distribution and numbers of isolates tested		
		Total #	Wild- Types	Mutants
		<u>Tested</u>		
	Aeromonas hydrophila group	15	15	0
5	Citrobacter freundii	48	35	13
	Enterobacter aerogenes	25	19	6
10	Enterobacter agglomerans	4	4	0
	Enterobacter cloacae	30	20	10
	Morganella morganii	25	19	6
	Proteus vulgaris	20	18	2
15	Providencia rettgeri	16	16	0
	Providencia stuartii	16	10	6
	Pseudomonas aeruginosa	32	26	6
	Other Pseudomonas spp.	17	4	13
	Serratia liquefaciens	20	20	0
	Serratia marscescens	25	20	5
20	Xanthomonas maltophilia	6	4	2
	Total	299	230	69
Group 2 Other Stains Tested:		<u>Total #</u> <u>Tested</u>		
25	Acinetobacter spp.	14		
	Citrobacter amalonaticus	12		
	Citrobacter diversus	13		
	Escherichia coli	25		
	Klebsiella oxytoca	20		
	Klebsiella pneumoniae	20		
30	Proteus mirabilis	24		
	Salmonella/Arizona	6		
	Salmonella paratyphi	4		
	Salmonella typhi	6		
	Shigella spp.	16		
35	Total	160		

Table 3Induction of  $\beta$ -lactamases on the Rapid  $\beta$ -lactamase Screen

Percent fluorescence at each concentration of antibiotic

	5	Ceftazidime (mcg/ml)					
		1	2	4	8	16	32
Ent. cloacae #498 (mut.)							
		Caz alone	96	90	100	97	100
		Caz w/Cfx	100	100	100	100	98
Ent. cloacae #20-8 (w.t.)							
		Caz alone	5	5	5	4	4
		Caz w/Cfx	47	7	5	5	4
Ps. aeruginosa #55-9 (w.t.)							
		Caz alone	24	8	7	5	6
		Caz w/Cfx	69	37	11	8	8
Ticarcillin (mcg/ml)							
		8	16	32	64		
Ent. cloacae #543 (mut.)							
		Ti alone	100	100	100	90	
		Ti w/Cfx	100	100	100	83	
Cit freundii #539 (w.t.)							
		Ti alone	15	15	14	12	
		Ti w/Cfx	89	75	67	26	
Ent. cloacae #20-8 (w.t.)							
		Ti alone	4	4	5	3	
		Ti w/Cfx	88	37	8	3	

Legend: Caz = ceftazidime; Caz w/Cfx = ceftazidime plus cefoxitin; Ti = ticarcillin; Ti w/Cfx = ticarcillin plus cefoxitin; w.t. = wild-type strain; mut = mutant strain.

Table 4

Number of Strains Induced in Each Antibiotic Containing Cefoxitin  
Group 1, Class I  $\beta$ -lactamase, Producers

	Organisms:	Total # Strains	NUMBER OF STRAINS INDUCED				
			Ti & Caz	Ti Only	Caz Only	Total	K-B
5	Cit. freundii	48	11	5	1	17	15
	Ent. cloacae	30	12	0	3	15	14
	Ps. aeruginosa	32	1	0	15	16	15
10	Ent. aerogenes	25	4	3	1	8	6
	Prt. vulgaris	20	1	0	1	2	9
	Mor. morganii	25	2	0	0	2	15
	Ser. marcescens	25	0	0	0	0	15
	Ent. agglomerans	4	0	0	0	0	0
15	Prv. rettgeri	16	0	0	0	0	0
	Prv. stuartii	16	0	0	0	0	2
	Aeromonas hydrophila grp	15	0	0	0	0	13
	Ser. liquefaciens	20	0	1	0	1	1
20	Xan. maltophilia	6	1	1	2	4	0
	Other Pseudomonads	17	0	1	2	1	4
		299	32	11	25	68	109
	Group 2						
25	Kleb. pneumoniae	20	0	0	0	0	0
	E. coli	25	0	0	0	0	0
	Kleb. oxytoca	20	0	0	0	0	0
	Prt. mirabilis	24	0	0	0	0	0
	Cit. amalonaticus	12	0	0	0	0	0
	Cit. diversus	13	0	0	0	0	0
30	Salmonella/Arizona	6	0	0	0	0	0
	Sal. typhi	6	0	0	0	0	0
	Sal. paratyphi	4	0	0	0	0	0
	Shigella spp.	16	0	0	0	0	0
	Acinetobacter spp.	14	0	0	0	0	0
35		160	0	0	0	0	0

What is claimed is:

1. An assay for rapid determination of beta-lactam antibiotic resistance in a bacterial strain

5 comprising

incubating the said bacterial strain in growth media containing an inducing beta-lactam antibiotic, and a second indicator noninducing beta-lactam antibiotic; and

10 measuring the extent of growth of the said bacterial strain during the said incubation.

2. An assay for rapid determination of beta-lactam antibiotic resistance in a bacterial strain comprising

15 growing the said bacterial strain in growth media containing an inducing antibiotic selected from the group consisting of cefoxitin, imipenem, sulbactam, and clavulanic acid, and a second indicator antibiotic selected from the group consisting of the penicillins, cephalosporins, and 20 cephemycins, and

measuring the growth of the said bacterial strain.

25 3. The assay of claims 1 or 2 wherein the said indicator antibiotic is ticarcillin, ceftazidime, mezlocillin, piperacillin, carbenicillin, ceftriaxone, and combinations thereof.

4. The assay of claims 1 or 2 wherein growth of the said bacterial strain is measured by fluorescence determination.

30 5. An assay for rapid determination of beta-lactam antibiotic resistance in a bacterial strain comprising

35 growing the said bacterial strain in growth media containing an inducing antibiotic, an indicator

antibiotic, and a metabolizable fluorogenic compound, and

measuring the growth of the said bacterial strain by detecting the release of a fluorophor from the said metabolizable fluorogenic compound.

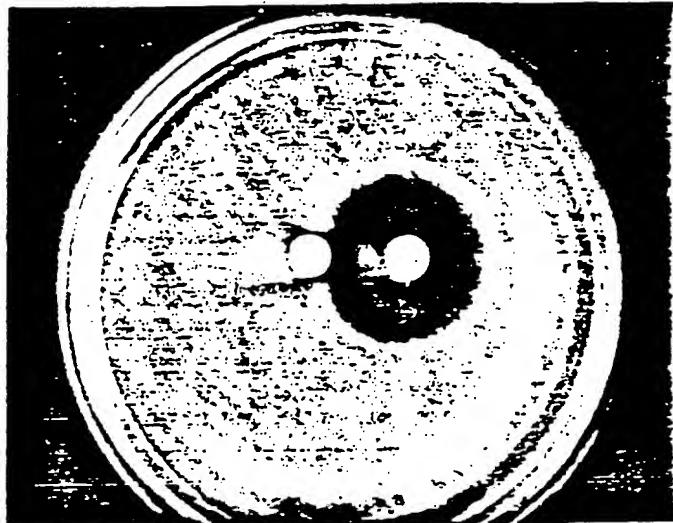
5 6. The assay of claim 4 wherein the fluorogenic compound is selected from the group consisting of 7-amido-4-methyl-coumarin and 4-methylumbellif erone compounds.

10 7. The assay of claim 4 wherein the inducing antibiotic is selected from the group consisting of cefoxitin, imipenem, sulbactam, clavulanic acid, and combinations thereof.

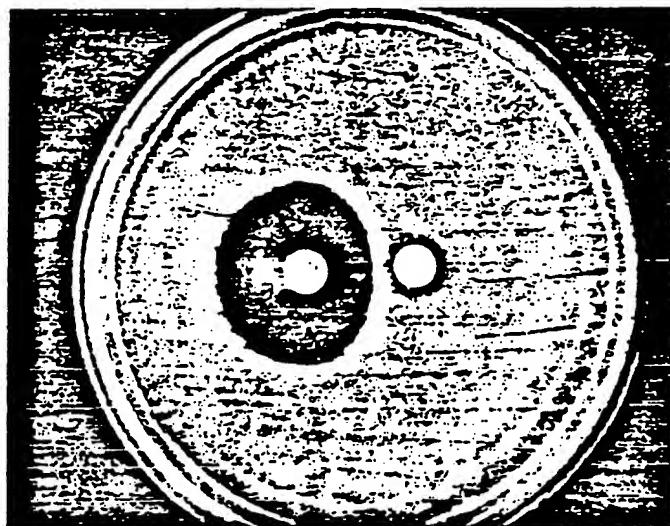
15 8. The assay of claim 4 wherein the indicator antibiotic is selected from the group consisting of a penicillin, a cephalosporin, a cephemycin, and combinations thereof.

9. A kit for determining the beta-lactam resistance of bacteria contained in a patient sample comprising

20 one or a plurality of incubation vessels containing a first dried reconstitutable inducing beta-lactam antibiotic, a second dried reconstitutable indicator noninducing antibiotic, and a metabolizable fluorogenic compound.



1A.



1B.

Figure 1.

- 1a) A typical pattern of inhibition around the mezlocillin (Mz) disk.
- 1b) Enterobacter cloacae showing constriction of the zone of inhibition between the Mz and the cefoxitin (FOX) disks.

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US91/03636

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) C12Q 1/02, 1/18, 1/20; C12N 1/38  
US CL 435/29, 32, 33, 244

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 32, 33, 244

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

search terms: fluorescence, beta lactam, antibiotic susceptibility

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US. A. 3,509,026 (Sanders et al) 28 April, 1970. See entire document.	1-9
Y,P	US. A 5,064,756 (Carr et al) 12 November 1991. See entire document.	1-9*
Y	Antimicrobial Agents and Chemotherapy. Volume 15, Number 6, SANDERS et al, "Emergence of Resistance to Cefamandole: Possible Role of Cefoxitin-Inducible Beta-Lactamases," pages 792-797, see entire document.	1-9

<input type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
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Date of the actual completion of the international search

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